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(71) Applicant (for all designated States except US): VIRA-GEN INCORPORATED [US/US]; 865 SW 78th Avenue, Suite 100, Plantation, FL 33324 (US).

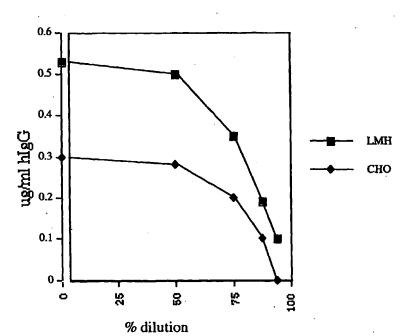
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): JERVIS, Karen

[GB/GB]; Viragen Scotland Ltd, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH25 0PZ (GB). ROBERTSON, Colin [GB/GB]; Viragen Scotland Ltd, Pentlands Business Park, Bush Loan, Penicuik, Midlothian EH25 0PZ (GB). CONNER, Joe [GB/GB]; School of Biological & Biomedical Science, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA (GB). STIMSON, William [GB/GB]; Department of Immunology, Strathclyde University, Glasgow G4 0NR (GB).

- (74) Agent: MURGITROYD & COMPANY; European Patent and Trade Mark Attorneys, 165-169 Scotland street, Glasgow G5 8PL (GB).
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[Continued on next page]

(54) Title: EXPRESSION OF MODIFIED ANTIBODIES IN AVIAN CELLS



(57) Abstract: The present invention relates to construct and methods which allow the expression of immunoglobulins or functional fragments thereof which have been altered so that they are humanised. The expression of immunoglobulins or fragments thereof takes place in avian cells, and the constructs used have been altered such that the expression levels in avian cells are higher than what would have been expected by simply using a humanised construct. The alterations are based on changing codons so that each amino acid of the codon that is used is the one which is most often found in avians.

Concentrations of human $\lg G$ in culture medium from cells transiently transfected with $4 \lg p 7.2$

Concentration of chimaeric R24 minibody was determined by human IgG1 ELISA. For % dilution, 0 = undiluted medium

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Expression of modified antibodies in avian cells

2

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3 The present invention relates to the expression of immunoglobulins, or more specifically, antibodies which have been altered so that the antibody is 'numanised', in avian cells. The antibody expression may occur either in vivo or in vitro. In the following document, the terms immunoglobulin and antibody are used interchangeably. 9 10 Antibodies are proteins of the immunoglobulin class which 11 are produced on exposure to an antigen. The antibody 12 produced recognises that antigen , binding selectively to it. There are five classes of immunoglobulin and the 13 14 following text relates primarily to antibodies of the 15 class IgG, although the other classes: IgA, IgD, IgY, IgE 16 and IgM are also included. An antibody molecule is made 17 up of two identical heavy chains linked by disulphide 18 bonds and two identical light chains. The biological effector functions of an antibody molecule derive from 19 the properties of a constant region, which is identical 20 for antibodies of all specificities within a particular 21 22 class. It is the variable region that contains the

site/s which allows binding to a particular epitope and

2

there is a variable domain at the end of each of the

- 2 heavy and light chains. These variable domains are
- 3 followed by a number of constant domains: Thus, the
- 4 binding of an antibody to an antigen occurs through
- 5 interactions of the variable domains of each pair of
- 6 heavy and light chains. Specifically, binding occurs in
- 7 the areas of the variable regions where there is most
- 8 variability. These regions are known as hyper variable
- 9 regions or complementarity determining regions (CDRs).

10

- 11 Generally, with the exception of vaccinations, antibodies
 - 12 are generated from a non-human source such as a mouse.
 - 13 When used in human therapeutic applications, such
 - 14 antibodies are usually recognised as foreign by the
 - 15 immune system. This results in human anti-mouse
 - 16 antibodies being produced, which may reduce the
 - 17 therapeutic effect of the initial antibody or produce
 - 18 undesirable side effects. Techniques have been developed
 - 19 which allow the base of murine antibodies plus those of
 - 20 other species to be manipulated in a way that the
 - 21 original antigen specificity is retained, but all the
 - 22 non-essential parts of the immunoglobulin sequence are
 - 23 replaced with the equivalent human derived sequence.
 - 24 This is known as 'humanising' an antibody. By using ...
 - 25 humanised antibodies, immunagenic responses are largely
 - 26 or highly avoided and effector functions improved.

- 28 However, even when an immunoglobulin sequence is
- 29 humanised there are still problems with the glycosylation
- 30 pattern of immunoglobulins for use in humans which have
- 31 not been produced in humans. IgG contains a conserved N-
- 32 glycosylation site located within the CHZ domain of each
- 33 heavy chain. The glycosylation pattern of

3

1 immunoglobuline is highly heterogeneous and has been

- 2 shown to have a significant effect on the biological,
- 3 pharmacological and physiochemical properties of the
- 4 immunoglobulin and include stability of the antibody and
- 5 half-life, tolerance in patient treatment and
- 6 interactions with complement components and other Ig
- 7 receptors. Alterations in the glycosylation pattern of
- 8 IgG has been linked to symptoms of rheumatoid arthritis.
- 9 The oligiosaccharides are produced in the Golgi apparatus
- 10 of the cellular interior and is regulated largely by the
- 11 glycosyltransferases present in this organelle.
- 12 Currently, antibodies for therapeutic applications are
- 13 being produced in a variety of cell lines and
- 14 transgenically, but many of these systems are not
- 15 particularly suitable in terms of glycosylation. The
- 16 same antibody produced in different cell lines and
- 17 animals may therefore be afforded different
- 18 characteristics which may result in differing functions
- 19 and pharmacokinetics. The expression of
- 20 glycosyltransferases differs with different cell types,
- 21 with the result that the glycosylation pattern of the
- 22 protein produced differs from that produced by other cell
- 23 types. In particular, it has been noted that non-human
- 24 mammalian cell lines, for example hamster cell lines,
- 25 show markedly different glycosylation patterns to humans
- 26 and therefore are likely to cause problems with
- 27 immunogenicity. It is known that normal Chinese Hamster
- 28 Ovary (CHO) Cells, which are the standard used in the
- 29 industry for the manufacture of recombinant proteins, do
- 30 not express the enzyme N-acetylglucosaminyltransferase-
- 31 III (GlcNAcT-III), which has the role of synthesising
- 32 carbohydrates which contain GlcNAc (Campbell & Stanley,
- 33 1984). Since GlcNAc is expressed in human immunoglobulin

1 G, it is known that human pr teins expressed in CHO cells

- 2 are not glycosylated in this manner. It is also
- 3 interesting to note that chicken IgG's also possess this
- 4 oligiosaccharide. It has been found by experts in the
- 5 area, such as that reported by Raju et al. 2002, that the
- 6 glycosylation patterns of avian cells are far more
- 7 similar than non-human mammalian glycosylation patterns
- 8 to human glycosylation patterns. :It therefore can be
- 9 seen that it would be highly advantageous to be able to
- 10 utilise chicken cells as a mode of production for
- 11 humanised recombinant proteins, compared to production of
- 12 such proteins in the standard mammalian cells.

13

- 14 US Patent No US5225539 entitled "Recombinant Altered
- 15 Antibodies and Method of Making Altered Antibodies"
- 16 describes a method of replacing the complementarity
- 17 determining regions of the heavy or light chain variable
- 18 domains of the receiving antibody with the corresponding
- 19 complementarity determining regions of a different
- 20 antibody with a different specificity. This method,
- 21 known as 'CDR grafting' is not the only method to produce
- 22 humanised antibodies, but is the most well known to those
- 23 knowledgeable in the field. The present invention
- 24 relates to antibodies which have been manipulated in any
- 25 manner which has the result of producing an antibody
- 26 which is more human-like in sequence than the wild-type
- 27 sequence.

- 29 However, although Patent No US5225539 describes a general
- 30 method which allows altered antibodies to be produced,
- 31 the methods described are directed towards production of
- 32 the altered antibodies in mammalian cells and does not
- 33 envisage the problems and advantages that arise when

5

1 producing antibodies in avian cells using this

2 technology.

3

4 While the methodology described in Winters' Patent

- 5 US5225539 is extremely useful for the production of CDR
- 6 grafted antibodies in mammalian cells, it can be seen
- 7 that it would also be useful to be able to easily produce
- 8 such CDR grafted and humanised antibodies in avian cells.
- 9 The production of such antibodies in avian cells would
- 10 have a significant advantage over the production in
- 11 mammalian cells if they are to be used as a human
- 12 therapeutic. This is because the glycosylation pattern
- 13 of human antibodies is more similar to avian antibodies
- 14 than the glycosylation of mammalian antibodies. As
- 15 previously mentioned, it is known that the glycosylation
- 16 pattern can have a significant effect on the bloactivity;
- 17 immunogenicity and therefore tolerance to the treatment
- 18 and also the pharmacokinetics of the antibody itself, and
- 19 therefore it would be extremely useful to produce
- 20 antibodies for human use of which the glycosylation
- 21 pattern is close to that of human antibodies.

- 23 It is also known that the yield of proteins produced in
- 24 non-avian transgenic animals, can be limited. It is
- 25 common practice to have to alter the culture conditions
- 26 or use expression vectors in order to obtain commercially
- 27 viable expression levels. The inventors have shown that
- 28 the expression of such proteins, especially
- 29 immunoglobulins and fragments thereof, in avian cells is
- 30 in general, higher than that observed in non-avian
- 31 animals. It can therefore be seen that it would be useful
- 32 and more time and cost-effective to produce antibodies
- 33 for human therapeutic use in avian cells. Figure 1 shows

3

- 1 the result of a Western blot to compare the expression
- 2 levels of the R24 protein in Chicken Hepatocellular (LMH)
- 3 cells, as compared to expression in Chinese Hamster Ovary
- 4 (CHO) cells. Figure 2 illustrates a comparison between
- 5 R24 protein produced in LMH and CHO cells, analysed by
- 6 human IqG1 ELISA. It can be seen that there is a
- 7 significantly higher level of protein produced in the LMH
- 8 cells as compared to the CHO cells. The inventors
- 9 believe that this is due to certain differences between
- 10 the translational machinery of the cell types in that the
- 11 LMH cells are more efficient in post-translationally
- 12 modifying the protein, than mammalian cells. This
- 13 belief is emphasised when the RNA message produced by
- 14 both cell types is analysed by PCR gel and the levels
- 15 produced by both types are similar.

16

. ;

- 17 One of the major problems of producing modified
- 18 antibodies in avian cells is that the codon usage in
- 19 avians differs from that in humans. Therefore, the
- 20 methodology described in US Patent No 5225539 is not
- 21 sufficient to allow the making of altered antibodies in
- 22 avian cells efficiently.

- 24 There is also the problem of actually producing that
- 25 which is coded for in a genetic construct in avian cells.
- 26 US Patent No US4816397 describes methods of producing
- 27 multi-chain polypeptides or proteins generally, however
- 28 again the methods are discussed mainly with regard to
- 29 mammalian cells and do not address the problems which
- 30 occur when producing multi-chain polypeptides, such as
- 31 antibodies, in avian cells. It should be noted that
- 32 there are a number of Patents and Patent Applications
- 33 which address the problem of producing basic single chain

l proteins in avian cells, however in these cases they do

- 2 not refer to the production of complex multi-chain
- 3 polypeptides, such as antibodi s, which pose their own
- 4 problems.

5

- 6 It can therefore be seen that it would be beneficial to
- 7 provide a method of producing and expressing modified
- 8 antibodies which have similar glycosylation patterns to.
- 9 those naturally produced in humans or more similar than
- 10 the glycosylation patterns obtained from antibodies from
- 11 culture in mammalian cells.

12

- 13 It can also be seen that it would be beneficial to
- 14 provide a method of producing and expressing modified
- 15 antibodies in avian cells, as specifically producing and
- 16 expressing humanised antibodies in avian cells, so that
- 17 they can be used as human therapeutics. ...

18

- 19 It would also be extremely useful if the expression of
- 20 the modified antibodies could be specifically in the egg
- 21 of a genetically modified avian, so that the antibody can
- 22 easily be collected and purified.

23

- 24 It is therefore a first object of the present invention
- 25 to provide antibodies which have a glycosylation pattern
- 26 which is more similar to naturally occurring human
- 27 glycosylation patterns than those antibodies produced in
- 28 mammalian cells.

29

- 30 It is a further object of the present invention to
- 31 provide a method of expressing such humanised antibodies
- 32 in avian cells in vitro and in vivo.

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8 It is a further object of the present invention to provide a method of xpressing humanised antibodies in 2 avian cells which are known to produce higher yields than observed in non-avian cell systems. 4 6 It is a further object of the present invention to provide a method of expressing humanised antibodies in 7 avian cells in vitro and in vivo. 8 9. It is a yet further object of the present, invention to 10 provide antibodies for therapeutic use, which have 11 glycosylation patterns which are more similar to those 12 naturally occurring in humans, than the glycosylation 13 14 patterns observed in antibodies produced in mammalian cells. 15 16 17 A still further object of the present invention is to 18 provide a construct which can be delivered into avian. cells, which will allow the production of antibodies or 19 20 humanised antibodies. 21 A final object of the present invention is to provide a 22 23 method of expressing humanised antibodies in avian cells, 24 so that they are specifically produced in the egg white 25 or egg yolk of an avian... 26 27 According to a first aspect of the present invention, 28 there is provided a DNA construct which when transfected into an avian cell will allow the production of an 29 30 antibody molecule or functional fragment of said

molecule, and which comprises at least one sequence which

comprises the variable domain of an immunoglobulin heavy

chain and at least one sequence which comprises the

9

- l variable domain of an immunoglobulin light chain, and
- 2 wherein the DNA construct is based on a non-avian
- 3 sequence and one or more of the codons in the DNA
- 4 construct have been altered such that for the amino acid
- 5 being encoded, the codon used is that which most
- 6 frequently appears in avians.

7

- 8 Preferably, the construct also contains an avian signal
- 9 peptide sequence.

10

- 11 Preferably the construct is cloned into a viral vector .
- 12 such as a lentivirus vector

13

- 14 Most preferably, the avian signal peptide sequence is a
- 15 signal peptide sequence from an egg white protein such as

٠, ١

16 lysozyme, ovalbumin, ovatransferrin or ovamucoid.

17

- 18 Most preferably, the construct also includes
- 19 immunoglobulin constant regions for dimerisation and
- 20 recruitment of effector functions.

21

- 22. Most preferably, the immunoglobulin constant regions are
- 23 CH2 and CH3 of any IgG class.

24

- 25 Still more preferably, the immunoglobulin constant
- 26 regions are human constant regions in order to provide a
- 27 humanised antibody.

28

- 29 Preferably, the construct may be transfected into an
- 30 avian cell using lipofection.

- 32 Alternatively, the construct is transfected into an avian
- 33 cell using electroporation.

10

1 2 A further option is that the construct may be directly 3 injected into the nucleus of an avian, into the germinal disc of an oocyte. 5 Preferably, codon usage in the construct is maximised for 6 7 those codons most frequently appearing in avians. 8 is, each codon is altered so that it still codes for the same amino acid, but uses the codon most often found to 9 10 code for that amino acid in avians. 11 According to a second aspect of the present invention, 1.2 13 there is provided an avian cell, containing the construct 14 of the first aspect, which expresses an immunoglobulin 15 molecule or functional fragment of said molecule. 16 . 17 Preferably the expressed immunoglobulin molecule or fragment thereof shows an avian glycosylation pattern. 18 19 Preferably the immunoglobulin or fragment thereof is 20 expressed at a higher expression level than a standard 21 22 human construct or humanised construct. 23 24 According to a third aspect of the present invention. 25 there is provided a method for producing avian cells 26 capable of expressing an immunoglobulin molecule or 27 functional fragment of said molecule, comprising 28 transfecting an avian cell with the DNA construct of the 29 first aspect. 30

Preferably the avian cell is a chicken cell, but may also be duck, turkey, quail, or ostrich.

11

- According to a fourth aspect of the present invention there is provided an immunoglobulin or functional fragment thereof produced using the method of the third aspect. 5 According to a fifth aspect of the present invention 6 there is provided a transgenic avian, which expresses the construct of the first aspect. 9 Preferably the antibody molecule, or functional fragment 10 of said molecule, that is coded for by the construct is 11 expressed in an egg of the transgenic avian. 12 13 Most preferably the construct is expressed in the egg 14 15 white. 16 Alternatively the construct is expressed in the egg yolk. 17 18 Preferably the immunoglobulin shows an avian 19 glycosylation pattern. 20 21 The present invention will now be illustrated, by way of . 22 example only, with reference to the following Figures in 23 24 which: 25 Figure 1 shows a Western blot showing the differences in 26 protein expression between chicken and mammalian cells; 27 28 and 29 Figure 2. is a graph illustrating the differences between
 - 30 Figure 2. is a graph illustrating the differences between 31 protein expression levels in chicken and mammalian cells, 32 as analysed by ELISA; and

Figure 3 is a table giving the frequency of codon usage 1 in chickens. 2 3 And with reference to the following sequences in which; 5 SEQUENCE ID 1 is the sequence of human IgG Fc used for 6 construction of chimeric and humanised minibodies; and 7 8 SEQUENCE ID 2 is the sequence of the chickenised IgG Fc 9 10 DNA sequence. 11 SEQUENCE ID 3 is the nucleotide alignment of the 12 original and chickenised human IgG Fc. 13 14 SEQUENCE ID 4 is the amino acid alignment of original and 15 chickenised human IgG Fc. 16 17 SEQUENCE ID 5 shows the chickenised R24 nuclectide 18 19 sequence. 20 SEQUENCE ID 6 shows the complete chickenised nucleotide 21 sequence of the R24 chimeric minibody 22 23 Generating a Construct 24 25 In this example a construct is produced which allows a 26 humanised murine R24 antibody to be produced in chickens. 27 28 The DNA encoding the single chain variable fragment is 29 designed in silico so that it can then be directly 30 synthesised using standard methods. 31

33 The starting sequences are human Vh and Vl sequences

13

1 which may b obtained from human IgM antibody. Vh and Vl

- 2 complementarity determining regions (CDRs) of anoth r
- 3 immunoglobulin (which in this case is the murine R24
- 4 immunoglobulin) are identified by standard methods (e.g.
- 5 see Antibodies-Structure and Sequence at
- 6 http://www.bioinf.org.uk/abs) and the R24 CDRs are
- 7 swapped directly into the human immunoglobulin framework.

8

- 9 The 3' end of the Vh DNA sequence is linked to the 5' end
- 10 of the Vl DNA sequence by a (Gly4Ser), peptide linker, as
- 11 seen in SEQUENCE ID 1. Included at the 3' end of the Vi
- 12 sequence is a sequence encoding a Bam H1 restriction
- 13 site. This gives the humanised R24 sequence 1. An IgG1
- 14 leader sequence is linked to the 5' and of Vh with the
- 15 inclusion of an Eco RI restriction site.

- 17 To provide the constant region of the immunoglobulin 2,
- 18 human IgG1 CH2/CH3 (Fc) DNA is then cloned by RT-PCR from
- 19 RNA. The primers incorporate Bam HI and Sal I
- 20 restriction sites and can be seen in SEQUENCE ID 2. The
- 21 amplified DNA fragment is cloned directly following PCR
- 22 using the PCR cloning vector pGEM-T (Promega). E coli
- 23 DH5a cells are transformed with the ligated plasmid,
- 24 plated out on amp selection media and colonies screened
- 25 the following day by PCR with M13 vector primers.
- 26 Positive clones with the appropriately sized insert can
- 27 then be selected and plasmid DNA can be prepared and
- 28 inserts sequenced to confirm the presence of
- 29 immunoglobulin constant region DNA. The insert from one
- 30 positive DNA clone is removed by Bam HI/Sal I digestion
- 31 and ligated into pGEM 3Z (Promega), digested with the
- 32 same restriction enzymes. After transformation and
- 33 overnight growth on amp media, colonies are screened by

14

pcR with M13 vector primers and plasmid prepared from one
positive clone.

3

4 One ug of the R24 gene synthesis product 1 is digested

5 with EcoRI/BamHI and ligated into plasmid hFc digested

6 with the same enzymes. After transformation and overnight

7 growth on amp media, colonies are screened by PCR with

8 M13 vector primers and plasmids prepared from clones with

9 the appropriately sized insert.

10

11 The entire insert DNA is then removed from pGEM3Z by

12 digestion with the restriction enzymes 'EcoRI/ Sall and

13 ligated into the mammalian expression vactor pCIneo

14 (Promega) digested with the same enzymes. After

15 transformation and overnight growth on amp media,

16 colonies are screened by restriction digestion

17 (EcoRI/SalI) of plasmid preparations. Plasmids may be

18 sequenced to confirm the presence of the minibody genes.

19

20 The insert can then be used to form a construct for

21 insertion into an avian cell. The insert, comprising the

22 Vh/Vl CDRs transplanted into a human immunoglobulin

23 framework 1 along with immunoglobulin constant domains 2

24 is removed from the pCIneo vector by BqIII/SfiI

25 digestion. The fragment that is gained by this digestion

26 consists of;

27

28 a promoter/ enhancer 6,

29 an intron 4,

30 the minibody which comprises the R24 variable regions 1

31 and the CH2 and CH3 constant regions 2, and

32 a poly A tail 3

15

- 1 In order f r the construct to be suitable for expression
- 2 in an avian c 11, the immunoglobulin leader sequence is
- 3 exchanged for an avi n specific sequence such as the
- 4 lysozyme signal peptide sequence 5. Also, both the R24
- 5 variable section coding sequence 1 and the CH2/CH3
- 6 constant region coding sequence 2 are chickenised.

7 8

'Chickenising' a Construct

9

- 10 Chickenising is defined as the alteration of codon usage
- 11 such that it is maximised for those codons most
- 12 frequently used in chickens. For expression in
- 13 transgenic chickens the codons of constructs are
- 14 optimised for most frequent codon usage in chickens.
- 15 Rowever, it can be seen that the optimisation could be
- 16 for the most frequent codon usage of any avian species.

17

- 18 EXAMPLE
- 19 Chickenising the human IgG Fc DNA sequence
- 20 For expression in transgenic chickens the codons of the
- 21 chimaeric and humanised minibody versions of R24 are
- 22 optimised for most frequent codon usage in chickens
- 23 (Gallus gallus). A table detailing frequency of codon
- 24 usage was downloaded from http://www.kazusa.or.jp and 1s
- 25 reproduced in Figure 3.

- 27 For an example of how chickenisation is carried out, it
- 28 can be seen that the amino acid Valine is encoded by 4
- 29 different codons, GTG, GTA, GTT and GTC with GTG used
- 30 most frequently in chickens (46% GTG, 11% GTA, 19% GTT
- 31 and 23% GTC*). To chickenise the human IgG Fc DNA, all
- 32 Valine codons were converted to GTG. Lysine is encoded
- 33 by two different codons, AAG and AAA, with AAG used most

16

1 frequently in chickens (58% Vs 42%). All AAA codons in

- 2 the sequence were converted to AAG. Not all codons
- 3 required alteration. For example, the two codons for
- 4 aspartic acid, GAT and GAC are used with almost equally
- 5 (40% vs 52%) and are not changed during the
- 6 chickenisation.

7

- 8 Sequence ID 1 shows the codons for the original human IgG
- 9 Fc DNA sequence. Sequence ID 2 shows the chickenised
- 10 version of this. Sequence ID 3 shows an alignment of the
- 11 nucleotide sequences, a dot indicates a sequence match
- 12 and the missing dots show where the codons have been
- 13 altered. Sequence ID 4 is an alignment of the amino acid
- 14 sequences which show that despite the alterations to
- 15 various codons the amino acids are still 100% identical.
- 16 Sequence ID 5 and 6 show the chickenised R24 scFv
- 17 sequence and complete chickenised R24 minibody

. . .

18 respectively.

19

20 * figures as given in Figure 3 = 99%

21

22 Inserting the Construct into an avian Cell

23

- 24 There are a number of possible methods that can be used
- 25 for transfection of an avian cell with the construct.
- 26 Transfection can either be transient or stable.

27

- 28 In transient transfection, supercoiled plasmid containing
- 29 the gene of interest is introduced into the nucleus of
- 30 the target cells at high copy number for short periods of
- 31 time (usually 24-96 hrs). During transient transfections
- 32 the DNA does not integrate into the cellular chromosomes.

1 For stable transfection either linear or plasmid DNA can

17

- 2 be introduced into the target clls and will either
- 3 integrate into the chromosomes or be maintained as a
- 4 stable episome. Linear DNA is optimal for stable
- 5 integration but is taken up less efficiently than
- 6 supercoiled plasmid. Cells in which the DNA has
- 7 integrated or is maintained as a stable episome can be
- 8 distinguished by selectable markers. For transfections
- 9 with pCIneo, the plasmid carries the neomycin
- 10 phosphotransferase gene which confers registance to
- 11 aminoglycosides such as G418. Culturing cells in the
- 12 presence of G418 selects for those that carry the
- 13 integrated or episomal DNA.

14

- 15 A variety of methods are available for the introduction
- 16 of DNA into mammalian cells and these include calcium
- 17 phosphate coprecipitation (Graham, R.L. and van der Erb,
- 18 AJA (1973) Virology 52, 456.) and electroporation
- 19 (Andreason, G.L. and Evans, G.A. (1988) BioTechniques 6,
- 20 650; Shigikawa, K. and Dover, W.J., (1988) BioTechniques
- 21 6, 742) but these have largely been superseded by
- 22 cationic liposome-mediated transfection (Felgner, J. at
- 23 al (1993) J Tiss Cult Metho. 15, 63). Other compounds
- 24 known to mediate transfection of mammalian cells include
- 25 lipopolyamines (Remy, J-S, Sirlin, C., Vierling, P and
- 26 Behr, J-P. (1994) Bioconjugate Chem. 5, 647) and
- 27 dendrimers (Haensler, J. and Szoka, FC (Jr) (1993)
- 28 Bioconjugate Chem 4, 372.).

- 30 Cationic liposomes, lipolyamines and dendrimers coat the
- 31 DNA to be transfected and mediate 1ts passage through the
- 32 cell membrane. A variety of factors influence the
- 33 efficiency of transfection and these include cell type,

18

1 media type and presence of serum and antibiotics, amount

- 2 and quality of plasmid DNA and cytotoxicity of
- 3 transfection reagent. All of thes usually have to be
- 4 optimised for each cell type and plasmid construct.

5

- 6 Alternatively, zygotic injection of the construct could
- 7 also be used to incorporate the construct.

8

- 9 Alternatively the construct may be cloned into a viral
- 10 vector such as a lentivirus vector and such vectors are
- 11 commercially available. Lentiviruses as vectors have been
- 12 developed from slow retroviruses, such as equine
- 13 infectious anaemia virus (EIAV), feline immunodeficiency
- 14 virus (FIV) or Human Immunodeficiency Virus (HIV). The
- 15 significant advantage using a lentiviral vector is that
- 16 the virus will infect cells that are not dividing, which
- 17 is appropriate to certain cell types of the present
- 18 invention.

19

- 20 If the construct contains a promoter such as an egg white
- 21 protein signal peptide, transgenic avians can then be
- 22 produced which lay eggs with the antibody of interest in
- 23 the egg white.

24

- 25 The chickenised construct may also be designed for
- 26 insertion into a gene contained within a plasmid, for
- 27 example it may be designed for insertion into a lysozyme
- 28 gene contained within a plasmid. The ATG site on the
- 29 lysozyme gene in the plasmid is destroyed by creating a
- 30 SalI site so that the lysozyme protein is not expressed.
- 31 The chickenised construct, which has its own ATG can then
- 32 be cloned into the SalI site.

- 1 Various modifications may be made to the invention herein
- 2 described, without departing from the scope thereof. For
- 3 example, any appropriate immunoglobulin sequence may be
- 4 used and any appropriate avian species may be used in
- 5 place of chickens with the codon bias changing
- 6 appropriately.

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2

1

A DNA construct which when transfected into an avian cell will allow the production of an antibody 5 molecule or functional fragment of said molecule, 6 and which comprises at least one sequence comprising 7 the variable domain of an immunoglobulin heavy chain 8 9 and at least one sequence comprising the variable domain of an immunoglobulin light chain, and wherein 10 the DNA construct is based on a non-avian sequence 11 12 and one or more of the codons in the DNA construct have been altered such that for the amino acid being 13 14 encoded, the codon used is that which most

15 16

A DNA construct as claimed in Claim 1, wherein the
 construct also contains an avian signal peptide
 sequence.

frequently appears in avians.

20

21 3. A construct as claimed in the previous Claims,22 wherein the construct is cloned into a viral vector.

23

24 4. A construct as described in Claim 3, wherein the 25 viral vector is a lentivirus vector.

26

27 5. A DNA construct as claimed in Claims 2 to 4, wherein 28 the avian signal peptide sequence is a signal 29 peptide sequence from an egg white protein.

30

31 6. A DNA construct as described in Claim 5, wherein the 32 egg white protein is chosen from the list lysozyme, 33 ovalbumin, ovatransferrin or ovamucoid.

		•
1	7.	A DNA construct as claimed in any of the previous
2		Claims, wherein the construct also includes
3		immunoglobulin constant regions for dimerisation and
4		recruitment of effector functions.
5		
6	8.	A DNA construct as claimed in Claim 7, wherein the
7		immunoglobulin constant regions are CH2 and CH3.
8		
9	9.	'A DNA construct as claimed in Claims 7 or 8, whereir
10		the immunoglobulin constant regions are human
11		constant regions.
12		
13	10.	A DNA construct as described in any of the previous
14		Claims, wherein the construct may be transfected
15		into an avian cell using electroporation.
16		
17	11.	A DNA construct as claimed in Claims 1 to 9, wherein
18		the construct may be transfected into an avian cell
19		using lipofection.
20		
21 -	12.	A DNA construct as claimed in Claim 1 to 9, wherein
22		the construct may be directly injected into the
23	:	nucleus of an avian.
24 -		
25	13.	A DNA construct as claimed in Claim 12, wherein the
26		construct may be directly injected into the germinal
27		disc of an oocyte.
28		
29	14.	A DNA construct as described in any of the previous
30		Claims, wherein codon usage in the construct is
31		maximised for those codons most frequently appearing
32		in avians.
33		

1	15.	An avian cell containing the construct described in
2		Claims 1 to 15, which expresses an immunoglobulin
3		molecule or functional fragment of said molecule.
4		
5	16.	An avian cell as described in Claim 16, wherein the
6		expressed immunoglobulin molecule or functional
7		fragment thereof shows an avian glycosylation
8		pattern.
9		
LO	17.	An avian cell as described in Claims 16 or 17,
11		wherein the immunoglobulin or fragment thereof is
12	•	expressed at a higher expression level than a
13		standard human construct or humanised construct.
14		
15	18.	A method for producing avian cells capable of
16		expressing an immunoglobulin molecule or functional
17		'fragment of said molecule, comprising transfecting
18		a an avian cell with the DNA construct as described in
L 9		Claims 1 to 15.
20		
21	19.	Preferably the avian cell is selected from a list of
22		chicken cell, duck cell, turkey cell, quail cell or
23		ostrich cell.
24		
25	20.	An immunoglobulin or functional fragment thereof
26		which is produced using the method described in
27		Claims 19 and 20.
28		
29	21.	A transgenic avian which expresses the construct
30		described in Claims 1 to 15.
31		

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1 22. A transgenic avian as described in Claim 22, wherein 2 the molecule coded for by the construct is expressed 3 in the egg of the transgenic avian.

4

5 23. A transgenic avian as claimed in Claim 23, wherein the construct is expressed in the egg white.

7

8 24. A transgenic avian as described in Claim 23, wherein the construct is expressed in the egg yolk.

- 25. A transgenic avian as described in Claims 22 to 25,
 wherein the expressed immunoglobulin shows an avian
- 13 glycosylation pattern.

1/5

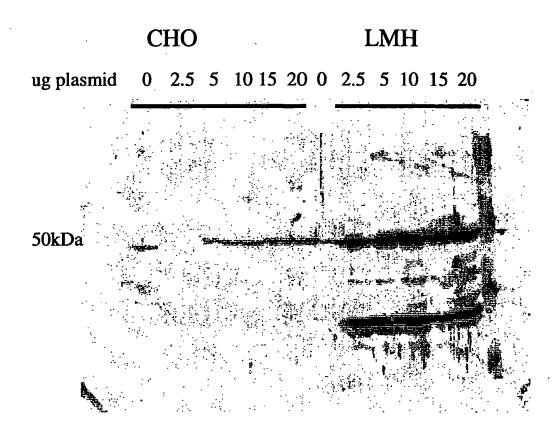


Figure 1: Equal numbers of cells were transfected with increasing amounts of chickenised R24 in pCIneo. Expression was detected by Western blotting using anti-human IgG Fc

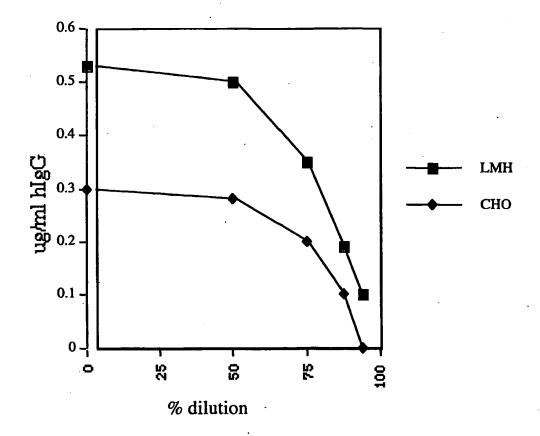


Figure 2: Concentrations of human IgG in culture medium from cells transiently transfected with 4ug p7.2

Concentration of chimaeric R24 minibody was determined by human IgG1 ELISA.

For % dilution, 0 = undiluted medium

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Figure 3: Gallus gallus [gbvrt]: 1867 CDS's (902768 codons)

A m A cid	Codon	Number	/1000	Fraction
Gly	GGG	15151.00	16.78	0.25
Gly	GGA	15334.00	16.99	0.26
Gly	GGT	10067.00	11.15	0.17
Gly	GGC	19197.00	21.26	0.32
Glu	GAG	39237.00	43.46	0.59
Glu	GAA	27671.00	30.65	0.41
Asp	GAT	21825.00	24.18	0.48
Asp	GAC	23834.00	26.40	0.52
Val	GTG	25842.00	28.63	0.46
Val	GTA	6430.00	7.12	0.11
Val	GTT	10831.00	12.00	0.19
Val	GTC	13180.00	14.60	0.23
A la	GCG	8155.00	9.03	0.13
A la	GCA	15732.00	17.43	0.24
A la	GCT	18019.00	19.96	0.28
A la	GCC	22576.00	25.01	0.35
Arg	AGG	10422.00	11.54	0.21
Arg	AGA	10268.00	11.37	0.21
Ser	AGT	9108.00	10.09	0.13
Ser	AGC	18604.00	20.61	0.27
Lys	AAG	32939.00	36.49	0.58
Lys	AAA	23618.00	26.16	0.42
Asn	AAT	14361.00	15.91	0.40
Asn	AAC	21629.00	23.96	0.60

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AmAcid	Codon	Number	/1000	Fraction
Met	ATG	21093.00	23.36	1.00
Ile	ATA	7094.00	7.86	0.17
Ile	ATT	14280.00	15.82	0.33
Ile	ATC	21332.00	23.63	0.50
Thr	ACG	7340.00	8.13	0.15
Thr	ACA	14212.00	15.74	0.28
Thr	ACT	11545.00	12.79	0.23
Thr	ACC	16795.00	18.60	0.34
Trp	TGG	10535.00	11.67	1.00
End	TGA	935.00	1.04	0.43
Cys	TGT	7336.00	8.13	0.37
Cys	TGC	12519.00	13.87	0.63
End	TAG	482.00	0.53	0.22
End	TAA	737.00	0.82	0.34
Tyr	TAT	10021	11.1	0.37
Tyr	TAC	17114	18.96	0.63
Leu	TTG -	10357.00	11.47	0.13
Leu	TTA	5406.00	5.99	0.07
Phe	TTT	13896.00	15.39	0.42
Phe	TTC	18856.00	20.89	0.58
Ser	TCG	4956.00	5.49	0.07
Ser	TCA	9525.00	10.55	0.14
Ser	TCT	11639.00	12.89	0.17
Ser	TCC	15048.00	16.67	0.22

5/5

A m A cid	Codon	Number	/1000	Fraction
Arg	CGG	8815.00	9.76	0.18
Arg	CGA	4502.00	4.99	0.09
Arg	CGT	4815.00	5.33	0.10
Arg	CGC	10528.00	11.66	0.21
Gln	CAG	29180.00	32.32	0.73
Gln	CAA	10558.00	11.70	0.27
H is	CAT	7845.00	8.69	0.37
H is	CAC	13525.00	14.98	· 0.63
Leu	CTG	34916.00	38.68	0.43
Leu	CTA	4886.00	5.41	0.06
Leu	CTT	9750.00	10.8	0.12
Leu	CTC	15185.00	16.82	0.19
Pro	CCG	7617.00	8.44	0.15
Pro	CCA	13515.00	14.97	0.26
Pro	CCT	12986.00	14.38	0.25
Pro	CCC	17062.00	18.90	0.33

1

2 Sequence ID 1. The sequence of human IgG Fc used for construction of chimaeric and humanised minibodies. AC CTT GCA GGA TCC GCA AGA CCC AAA TCT 7 TGT 8 9 GAC AAA ACT CAC ACA TGC CCA CCG TGC 10 CCA GCA 11 12 CCT GAA CTC CTG GGG GGA CCG TCA GTC 13 TTC CTC 14 15 TTC CCC CCA AAA CCC AAG GAC ACC CTC 16 ATG ATC 17 18 TCC CGG ACC CCT GAG GTC ACA TGC GTG 19 GTG GTG 20 21 GAC GTG AGC CAC GAA GAC CCT GAG GTC 22 AAG TTC 23 24 AAC TGG TAC GTG GAC GGC GTG GAG GTG 25 CAT AAT 26 27 GCC AAG ACA AAG CCG CGG GAG GAG CAG 28 TAC AAC 29 30 AGC ACG TAC CGG GTG GTC AGC GTC CTC 31 ACC GTC 32 33

- CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG 9 10 CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC 11 12 13 CCA TCC CGG GAG GAG ATG ACC AAG AAC CAG GTC 15 AGC CTG ACC TGC CTG GTC AAA GGC TTC 16 TAT CCC 17 18, AGC GAC ATC GCC GTG GAG TGG GAG AGC 19 AAT GGG 20 21 CAG CCG GAG AAC AAC TAC AAG ACC ACG 22 CCT CCC 23 24 GTG CTG GAC TCC GAC GGC TCC TTC TTC 25 CTC TAT 27 AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG 29
 - 30
 31 CAG GGG AAC GTC TTC TCA TGC TCC GTG
 32 ATG CAT

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1 GAG GCT CTG CAC AAC CAC TAC ACG CAG

2 AAG AGC

3

4 CTC TCC CTG TCC CCG GGT AAA TGA TAA

5 GTC GAC

6

7 ACG TGA TC

Sequence ID 2. The chickenised human IgG Fc DNA sequence.

Codon alterations are in red.

_

6 AC CTT GCA GGA TCC GCC AGA CCC AAG TC

7 TGC

8

9 GAC AAG ACC CAC ACA TGC CCA CCC TGC

10 CCA GCC

11

12 CCC GAG CTG CTG GGG GGA CCC TCC GTG

13 TTC CTG

14

15 TTC CCC CCA AAG CCC AAG GAC ACC CTG

16 ATG ATC

17

18 TCC CGC ACC CCC GAG GTG ACA TGC GTG

19 GTG GTG

20

21 GAC GTG AGC CAC GAG GAC CCC GAG GTG

22 AAG TTC

23

24 AAC TGG TAC GTG GAC GGC GTG GAG GTG

25 CAC AAC

26

27 GCC AAG ACA AAG CCC CGC GAG GAG CAG

28 TAC AAC

29

30 AGC ACC TAC CGC GTG GTG AGC GTG CTG

31 ACC GTG

32

33 CTG CAC CAG GAC TGG CTG AAC GGC AAG

34 GAG TAC

1 AAG TGC AAG GTG TCC AAC AAG GCC CTG CCA GCC 5 CCC ATC GAG AAG ACC ATC TCC AAG GCC AAG GGG CAG CCC CGC GAG CCA CAG GTG TAC ACC CTG CCC 10 CCA TCC CGC GAG GAG ATG ACC AAG AAC 11 CAG GTG 12 13 AGC CTG ACC TGC CTG GTG AAG GGC TTC 14 TAC CCC 15 16 17 AGC GAC ATC GCC GTG GAG TGG GAG AGC AAC GGG 18 19 CAG CCC GAG AAC AAC TAC AAG ACC ACC 20 CCC CCC 21 22 GTG CTG GAC TCC GAC GGC TCC TTC TTC 23 CTG TAC 24 25 AGC AAG CTG ACC GTG GAC AAG AGC AGG 26 TGG CAG 27 28 29 CAG GGG AAC GTG TTC TCC TGC TCC GTG ATG CAC 30 31 32 GAG GCC CTG CAC AAC CAC TAC ACC CAG AAG AGC

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3 GTG GAC

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   codons are shown in red. The underlined
   nucleotides encode the lysozyme leader amino
   acide
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   GGC CGG GTC GAC ATG AGG TCT TTG CTA
   ATC TTG
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   GTG CTT TGC TTC CTG CCC CTG GCT GCT
11
   CTG GGG
12
13
   GAT GTG CAG CTG GTG GAG TCC GGG GGA
   GGC CTG
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   GTG CAG CCC GGA GGG TCC CGC AAG CTC
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   TCC TGC
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   GCC GCC TCC GGA TTC ACC TTC AGC AAC
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   TTC GGA
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   ATG CAC TGG GTG CGC CAG GCC CCC GAG
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   AAG GGG
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   CTG GAG TGG GTG GGA TAC ATC AGC AGC
   GGC GGC
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   AGC TCC ATC AAC TAC GCC GAC ACC GTG
29
   AAG GGC
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31
   CGC TTC ACC ATC TCC AGA GAC AAC CCC
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   AAG AAC
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ACC CTG TTC CTG CAG ATG ACC AGC CTG AGG TCC .3 GAG GAC ACA GCC ATC TAC TAC TGC ACC AGA GGG GGA ACC GGG ACC AGA TCC CTG TAC TAC . 7 TTC GAC ٠9 TAC TGG GGC CAG GGC GCC ACA CTG ATC 10 GTG TCC 11 12 TCC GGG GGA GGC GGC TCC GGG GGA GGC 13 GGC TCC. 14 15 GGG GGA GGC GGC TCC GAT ATC CAG ATG ACA CAG 17 18 ATC ACA TCC TCC CTG TCT GTG TCT CTG 19 GGA GAC 20 21 AGA GTG ATC ATC AGC TGC AGG GCT AGC 22 CAG GAC 23 24 ATC GGC AAT TTT CTG AAC TGG TAC CAG CAG GAA 26 27 CCA GAT GGA TCT CTG AAG CTG CTG ATC 28 TAC TAC 29 30 ACA TCT AGA CTG CAG TCC GGA GTG CCA 31 TCC AGG 32

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	14/19	•

TTC AGC GGC TGG GGG TCT GGA ACA GAT
TAC TCT

ACC ACC ATT AGC AAC CTG GAG GAA GAG
GAT ATC

GCC ACC TTC TTC TGC CAG CAG GGC AAG
ACA CTG

CCC TAC ACC TTC GGA GGG GGG ACC AAG
CTG GAG

ATC AAG CGC GGA TCC GCC GCC G

12

15/19

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Sequence ID6. The complete chickenised nucle tide
   sequence of the R24 chimaeric minibody.
   GGC CGG GTC GAC ATG AGG TCT TTG CTA
   ATC TTG
 6
   GTG CTT TGC TTC CTG CCC CTG GCT GCT
   CTG GGG
 9
   GAT GTG CAG CTG GTG GAG TCC GGG GGA
10
   GGC CTG
11
12
13 GTG CAG CCC GGA GGG TCC CGC AAG CTC
   TCC TGC
14
15
   GCC GCC TCC GGA TTC ACC TTC AGC AAC
16
   TTC GGA
17
18
   ATG CAC TGG GTG CGC CAG GCC CCC GAG
19
   AAG GGG
20
21
   CTG GAG TGG GTG GGA TAC ATC AGC AGC
   GGC GGC
23
24
   AGC TCC ATC AAC TAC GCC GAC ACC GTG
25
   AAG GGC
26
27
   CGC TTC ACC ATC TCC AGA GAC AAC CCC
28
   AAG AAC
29
30
   ACC CTG TTC CTG CAG ATG ACC AGC CTG
31
   AGG TCC
32
33
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1 GAG GAC ACA GCC ATC TAC TAC TGC ACC 2 AGA GGG 3 GGA ACC GGG ACC AGA TCC CTG TAC TAC TTC GAC 6 7 TAC TGG GGC CAG GGC GCC ACA CTG ATC GTG TCC TCC GGG GGA GGC GGC TCC GGG GGA GGC 10 11 GGC TCC 12 13 GGG GGA GGC GGC TCC GAT ATC CAG ATG 14 ACA CAG 16 ATC ACA TCC TCC CTG TCT GTG TCT CTG GGA GAC 17 18 19 AGA GTG ATC ATC AGC TGC AGG GCT AGC CAG GAC 20 21 22 ATC GGC AAT TTT CTG AAC TGG TAC CAG CAG GAA 23 24 25 CCA GAT GGA TCT CTG AAG CTG CTG ATC 26 TAC TAC 27 28 ACA TCT AGA CTG CAG TCC GGA GTG CCA TCC AGG 29 30 TTC AGC GGC TGG GGG TCT GGA ACA GAT 31

TAC TCT

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CTG ACC ATT AGC AAC CTG GAG GAA GAG
   GAT ATC
 2
 3
   GCC ACC TTC TTC TGC CAG CAG GGC AAG
   ACA CTG
 6
   CCC TAC ACC TTC GGA GGG GGG ACC AAG
   CTG GAG
  ATC AAG CGC GGA TCC GCC AGA CCC AAG
10
   TCC TGC
11
12
   GAC AAG ACC CAC ACA TGC CCA CCC TGC
   CCA GCC
14
15
   CCC GAG CTG CTG GGG GGA CCC TCC GTG
1.6
   TTC CTG
17
18
   TTC CCC CCA AAG CCC AAG GAC ACC CTG
19
   ATG ATC
20
21
   TCC CGC ACC CCC GAG GTG ACA TGC GTG
22
   GTG GTG
23
24
   GAC GTG AGC CAC GAG GAC CCC GAG GTG
25
  AAG TTC
26
27
   AAC TGG TAC GTG GAC GGC GTG GAG GTG
28
  CAC AAC
29
30
   GCC AAG ACA AAG CCC CGC GAG GAG CAG
31
   TAC AAC
32
33
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AGC ACC TAC CGC GTG GTG AGC GTG CTG ACC GTG CTG CAC CAG GAC TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTG TCC AAC AAG GCC CTG CCA GCC CCC ATC GAG AAG ACC ATC TCC AAG GCC 10 AAG GGG 11 12 CAG CCC CGC GAG CCA CAG GTG TAC ACC 13 CTG CCC 14 15 CCA TCC CGC GAG GAG ATG ACC AAG AAC 16 CAG GTG 17 18 AGC CTG ACC TGC CTG GTG AAG GGC TTC 19 TAC CCC 20 21 AGC GAC ATC GCC GTG GAG TGG GAG AGC AAC GGG 23 24 CAG CCC GAG AAC AAC TAC AAG ACC ACC 25 CCC CCC 26 27 GTG CTG GAC TCC GAC GGC TCC TTC TTC CTG TAC 29 30

28

AGC AAG CTG ACC GTG GAC AAG AGC AGG 31

TGG CAG 32

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1 CAG GGG AAC GTG TTC TCC TGC TCC GTG

2 ATG CAC

3

4 GAG GCC CTG CAC AAC CAC TAC ACC CAG

5 AAG AGC

6

7 CTC TCC CTG TCC CCC GGC AAG TGA TAA

8 GTC GAC

9

10 ACC TGA TC